氏　　名	カンディス　ジン　ティモネラ　タヒミック Candice Ginn Timonera Tahimic
学位の種類	博士（生命科学）
学位記番号	甲第５３号
学位授与年月日	平成１８年　３月１０日
学位授与の要件	学位規則第４条第１項該当
学位論文題目	Evidence for a role of Collapsin Response Mediator Protein-2 (Crmp-2) in signaling pathways that regulate the proliferation of non-neuronal cells （非神経系細胞の増殖を制御するシグナル伝達経路内における Collapasin Response Mediator Protein-2 (Crmp-2)の役割）
学位論文審査委員	（主査）　佐藤健三 （副査）　汐田剛史　　押村光雄

学位論文の内容の要旨

Collapsin response mediator protein-2 (Crmp-2), also known as Crmp-62/TOAD-64/Ulip2/DRP2, belongs to a family of phosphoproteins involved in neurite outgrowth and axonal guidance. It was recently reported that phosphorylation of Crmp-2 at threonine 514 by GSK3β regulates neuronal polarization and that overexpression of a non-phosphorylatable form of Crmp-2 or inhibition of GSK3β induced the formation of multiple axon-like neurites in hippocampal neurons. Moreover, phosphorylation of Crmp-2 at threonine-555 by Rho kinase has also been shown to regulate growth cone collapse through both Semaphorin IIIA-dependent and independent pathways. To date, most of our understanding on the biological significance of Crmp-2 has been in the context of its function in neurons. Although a few studies have also reported that Crmp-2 is also expressed in mitotically active, non-neuronal cells and was even observed to co-localize with microtubule structures the implications of Crmp-2 expression in these cells is poorly understood. This current study explores the biological significance of Crmp-2 and its phosphorylation in mitotically-active, non-neuronal cells.

方　法

Protein lysates from mouse tissue and fibroblastic cell lines were subjected to two-dimensional gel electrophoresis followed by Western blotting using an antibody against

Crmp-2. For fluorescence imaging studies, expression plasmids coding for EGFP fusions of wild type or mutant Crmp-2 cDNA were transfected into human and mouse cell lines. Fluorescent signals were visualized using a confocal microscope equipped with suitable filters. GSK3β inhibition was carried out by incubating fibroblasts at varying concentrations of LiCl. Rho kinase inhibition was performed using HA1077 or Y-27632.

結　果

Two dimensional electrophoresis followed by Western blotting revealed the existence of multiply phosphorylated forms of Crmp-2 in mouse fibroblasts and non-neuronal tissue. In HeLa cells, overexpression of wild type Crmp-2 resulted in nuclear blebbing and consequent cell death 48 hours after transfection. Cells overexpressing a Crmp-2 mutant that lacked amino acids 413 to 572 exhibited altered localization of Crmp-2 and earlier onset of nuclear aberrations. Furthermore, overexpression of a T555A mutant of Crmp-2 wherein threonine 555 was replaced with the non-phosphorylatable amino acid, alanine also resulted in cell death 48 hours after transfection. In all cases, cells overexpressing Crmp-2 or its mutant forms were completely eliminated from culture even under selective conditions.

Using the GSK3β inhibitor, LiCl, it was demonstrated that phosphorylation of Crmp-2 at threonine 514 in fibroblasts was dependent on GSK3β activity. Furthermore, a drop in phosphorylated threonine 514 levels was observed as fibroblast cultures approached confluence. This dephosphorylation event was reversible; when confluent cultures were re-plated and allowed to grow in low cell density conditions, phosphorylated threonine 514 levels again increased. Reduced threonine 514 phosphorylation levels were also observed after serum starvation-induced quiescence. Re-addition of serum resulted in the restoration of phosphorylated threonine 514 levels comparable to that of pre-starvation levels.

Interestingly, hyperphosphorylation of Crmp-2 at threonine 555 was observed in an ATM mouse tumor and a derivative cell line. A previous study has shown that Rho kinase can phosphorylate Crmp-2 at threonine 555 in neurons. Nevertheless, treatment with the Rho kinase inhibitors, HA1077 and Y-27632 did not reduce threonine 555 phosphorylation levels in both non-tumor fibroblasts and tumor cell line.

考　察

This study has presented evidence for a non-neuronal function of Crmp-2, specifically in the control of cell proliferation, and that Crmp-2 function in mitotic, non-neuronal cells is effected through its phosphorylation by associated kinases. Similarities in the regulation of Crmp-2 phosphorylation in neurons and fibroblast could be observed, such as the responsiveness of Crmp-2 phosphorylation at threonine 514 to GSK3β activity. While the

interaction between GSK3β and Crmp-2 is important for the establishment of polarity in neurons, the findings of this current study suggest that the interaction between GSK3β and Crmp-2 is has implications in the establishment of cellular quiescence in mitotic, non-neuronal cells.

Results show a correlation between elevated threonine 514 phosphorylation levels and a state of active proliferation while hyperphosphorylation of threonine 555 was observed in a malignancy. Taken together, a scenario can be envisioned wherein hyperphosphorylation of certain amino acid residues of Crmp-2 may result in increased cellular proliferation, consequently leading to tumorigenesis.

The non-responsiveness of threonine 555 phosphorylation to Rho kinase inhibition, suggests that a yet to be identified kinase phosphorylates Crmp-2 in mitotic, non-neuronal cells. Alterations in the kinase activity of this unidentified protein may explain the hyperphosphorylation of Crmp-2 at threonine 555 and may have important implications in tumorigenesis.

結　論

This study has demonstrated that Crmp-2, a protein well-known for its role in the establishment of neuronal polarity, is also involved in signaling pathways that regulate the proliferation of non-neuronal cells. The involvement of Crmp-2 in these pathways is mediated via its phosphorylation by GSK3β and a yet to be identified kinase, and is independent of Rho kinase activity. Perturbations in the activity of associated kinases may lead to de-regulation of Crmp-2 phosphorylation levels and consequently, carcinogenesis.

審　査　結　果　の　要　旨

本研究は、非神経系細胞における Collapsin response mediator protein-2 (Crmp-2)の生物学的意義を検討したものである。正常またはC末端領域に突然変異を導入した Crmp-2 の過剰発現実験によって、このタンパク質の細胞内局在および細胞増殖に対する影響を観察している。さらにキナーゼ阻害実験と伴に免疫生化学的手法を用いて、Crmp-2 のリン酸化動態を解析した。その結果、次の 4 点が明らかとなった。(1) Crmp-2 の過剰発現により細胞毒性が観察される。(2) Crmp-2 のスレオニン 514 における脱リン酸化は細胞の接触阻害による増殖休止に一致しており、これが GSK3β活性に依存して起こる。(3) Crmp-2 のスレオニン 555 はいくつかのがん細胞株で過剰にリン酸化されている。(4) 線維芽細胞における Crmp-2 スレオニン 555 のリン酸化は、神経細胞で認められている Rho キナーゼではなく、異なる未知のキナーゼに依存する。この研究により、Crmp-2 リン酸化の制御の異常により異常増殖あるいはがん化が引き起こされる可能性が示唆され、Crmp-2 が神経細胞の極性形成・軸索伸長に働くだけでなく、非神経系細胞の増殖シ

グナル伝達経路内で関与することが明らかとなった。本論文の内容は、細胞増殖シグナル伝達、がんの分子マーカーへの応用などの研究分野において明らかに学術水準を高めたものと認める。